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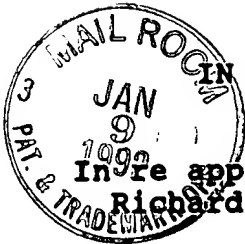
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PATENT  
Our Dock t: P31 8026



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:  
Richard H. Tullis

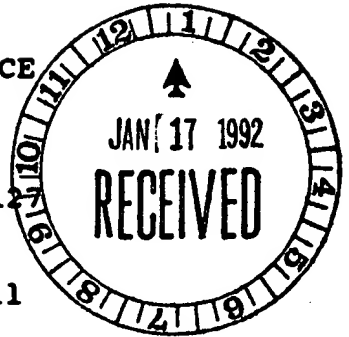
Serial No.: 140,916

Filed: December 29, 1987

For: OLIGONUCLEOTIDE  
THERAPEUTIC AGENT AND  
METHOD OF MAKING SAME

Group Art Unit: 12

Examiner: Martinell



Los Angeles, CA 90071  
November 16, 1988

DECLARATION UNDER 37 C.F.R. 1.131

Hon. Commissioner of  
Patent & Trademarks  
Washington, D.C. 20231

Sir:

I, Cathryn Campbell, residing at 11570 Caminito Gusto,  
San Diego, California 92131, declare as follows:

1. I received my Ph.D. from the University of  
California, Davis, in 1978 and my J.D. from the U.C.L.A.  
School of Law in 1983.

2. I am a member of the State Bar of California and  
admitted to practice before the United States Patent and  
Trademark Office.

1

Nov 16, 1988  
Cathryn Campbell  
Nov 16, 1988

3. I am presently counsel to the law firm of Pretty, Schröder, Bruggmann and Clark. Previously I was associated with the firms of Irell and Manella, and Fulwider, Patton, Rieber, Lee & Utecht.

4. I am an attorney of record in the above-identified application which is a continuation of Ser. No. 002,014, now abandoned, which was a continuation of Ser. No. 314,124, now abandoned.

5. The parent application, Ser. No. 314,124, was originally prepared and filed on October 23, 1981 by the Fulwider firm prior to my employment there. William M. Smith, Esq. was one of the attorneys who interfaced with the inventor of these applications, drafted the original application and filed it. I worked on the case after Mr. Smith left and, when I moved to Pretty, Schroeder, Brueggemann and Clark, the application and related files were transferred to me.

6. All pending claims were finally rejected in the parent application, Ser. NO. 002,014, as anticipated under 35 U.S.C. 102(e), or, alternatively, as obvious under 35 U.S.C. 103, over a patent to T'so. The application for the T'so patent was filed November 12, 1980.

7. I have recently reviewed various correspondence files relating to the parent S r. No. 314,124 application which were among those files transferred to me from the Fulwider firm. Among these files was one entitled "Tullis, Richard H., Review and Advise re Synthetic Oligonucleotides Anti Viral Agent." This file contains documents evidencing a date of conception for the invention which predates November 12, 1980. Copies of relevant pages contained therein which show conception of the invention disclosed and claimed in the above-identified application are appended hereto as Exhibits I, II and III. Although the dates on the Exhibits have been obliterated, I have reviewed them and all predate November 12, 1980.

8. The invention as disclosed and claimed in Ser. No. 314,124 and now claimed in Ser. No. 140,916 was conceived prior to November 12, 1980, the effective date of the T's reference. Attached in support of conception before this date are Exhibits I through III.

9. Exhibit I is a letter from the inventor, Richard H. Tullis, to William M. Smith, Esq. It was dated by Mr. Tullis prior to November 12, 1980. The letter sets forth the essential elements of the invention in sufficient detail to permit one skilled in the art to practice the invention without undue experimentation. On the last page, Mr. Tullis

states that "this idea first occurred to me .....in the afternoon of [a date preceding November 12, 1980]."

10. Exhibit II is a letter from the inventor Richard H. Tullis to William M. Smith, Esq. which was dated by the writer and stamped as received by the Fulwider firm. The letter discloses the invention. It was the policy of the Fulwider firm that correspondence received by the firm was opened by a mail clerk and stamped with the date received. Correspondence was then attached to the appropriate file. Both dates precede November 12, 1980. The letter expands at length on the specific details of the invention.

11. Exhibit III is an Information Sheet prepared by the Fulwider firm for use in opening a file. It lists as the subject matter "Synthetic Oligonucleotides Anti Viral Agent" and refers to data received as a letter with articles. The Information Sheet is dated prior to November 12, 1980.

12. The files in my possession contain evidence of the diligence of the attorneys and the inventor in filing the original application. Such evidence includes documents indicating extensive interaction between the attorneys and the inventor including memos of telephone conversations, indications of personal meetings, and drafts of applications. Various of these documents are identified and submitted

h rewith as Exhibits IV through VII.

13. Exhibit IV is a copy of a l t t r dat d November 6, 1980, from the inventor, Richard H. Tullis to William M. Smith, Esq., which accompanied the disclosure materials and instructed Mr. Smith to "fire away". Certain material unrelated to the date of the letter has been obliterated.

14. Exhibit V is a copy of a letter from Richard H. Tullis to William M. Smith, Esq. dated February 4, 1981, accompanying a first draft of the patent. Dr. Tullis indicates that much work on the patent "remains to be done." Certain material unrelated to the date has been obliterated.

15. Exhibit VI is a letter from Richard H. Tullis to William M. Smith, Esq., dated February 23, 1981, accompanying literature on the invention. Certain material unrelated to the date has been obliterated.

16. Exhibit VII is a letter from Richard H. Tullis to William M. Smith, Esq., dated June 1, 1981, accompanying a revised draft of the application and indicating that it is still incomplete.

17. The file contains other letters, documents, telephone memos indicating a continuing interaction between

Dr. Tullis and Mr. Smith vouching the extensive work directed towards preparation and filing of the application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

Dated: Nov 16, 1988

  
CATHRYN CAMPBELL

Enc:

Ex I - Letter  
Ex II - Letter  
Ex III - Information Sheet  
Ex IV - Letter  
Ex V - Letter  
Ex VI - Letter  
Ex VII - Letter

# California Biomedical Research Foundation

DEPARTMENT OF BIOLOGICAL CHEMISTRY

741 Arenas Street  
La Jolla, California 92037

Telephone (714) 454-8571

Dear Bill,

It was a pleasure talking to you on the phone about my idea for a new type of antibiotic employing synthetic oligonucleotides specific for particular mRNAs. Especially in finding that you had attended UCSD, knew Paul Price and had taught O-Chem with him. I am looking forward to meeting both you and Mr. Kovelman. Since it's getting late now I'll get down to business.

The idea is as follows:

① Synthetic oligodeoxynucleotides can be now manufactured which bind specifically to particular mRNAs, forming an RNA-DNA hybrid.

② mRNA-DNA hybrids cannot be translated into proteins

③ Oligonucleotides are taken up by cells and will form RNA-DNA hybrids under physiological conditions (ie 37 C; ca 0.2M salt)

Therefore it is possible to make specific oligonucleotides which will selectively block any selected organism from growing. (eg block a virus growing inside a cell)



This system is not relegated to viral infections alone. Any organism (such as an insect, a malarial parasite or a leprosy causing bacterium) which can be made to take up the dinucleotide can be selectively killed. Thus this system represents an entirely new class of antibiotics which can <sup>in theory</sup> be directed at any organism.

I have considered several possibilities with regard to this system which might occur as objections against it. I will deal with these in order of importance.

① Is this system sufficiently selective?

The best evidence experimentally is that a 12 base digodeoxynucleotide designed to specifically bind to gastrin mRNA can select it out of a population of about 30,000 different sequences which are present in stomach A<sup>+</sup> RNA!

Another way to view the same question is to ask how many possible sequences are there in any cell? In general there are about 10,000 different mRNA sequences per cell each about 1000 bases long. Thus the sequence complexity is about  $10^7$  bases or nucleotides (NT). For a 15 NT oligomer there are  $4^{15}$  different possibilities.

Thus the chance of a random matchup between ~~the~~ any particular mRNA and a specific 15 NT oligomer

$$\text{is } \frac{4^{15}}{10^7} = \frac{1.07 \times 10^9}{10^7} \approx 100:1$$

As you increase the size of the dinucleotide, the chance of a random hybridization decreases exponentially

$$4^{12} = 17 \times 10^6$$

$$4^{15} = 1.07 \times 10^9$$

$$4^{13} = 67 \times 10^6$$

$$4^{16} = 4.3 \times 10^9$$

$$4^{14} = 268 \times 10^6$$

$$4^{17} = 17 \times 10^9$$

Thus it is possible to design an oligonucleotide which is extremely specific for a particular mRNA, and therefore for a specific organism.

② Will the system work in a living situation?

The growth of RSV (Rous sarcoma virus) can be selectively blocked in cultured cells with no evidence of toxicity to the cells by a specific oligonucleotide. The treatment also blocks the ability of the virus to transform the cells into cancer cells.

③ What do I propose that's unique?

The design of particular oligodeoxynucleotides (synthesized as phosphotriesters) which inactivate organisms other than RSV. (eg malaria)  
Phosphotriester formation has two beneficial effects

④ it makes the dinucleotide virtually immune to degradation by the cell

⑥ it makes the oligonucleotide soluble in lipid phases (eg membranes) while preserving its ability to bind to RNA and form hybrids. This should allow it easy access to the interior of the cell, as shown in the Too paper.

I hope this short explanation is sufficient. I have done many other calculations, but I see no reason to go into them at this time. Please let me know what else you require.

Best Regards

Richard H. Jull.

PS.

PPS. This idea first occurred to me during a conversation with Ken Widder (Kendrew Biosystems) in Long Beach in the afternoon of . . . .

**F. Jander, Patton, Kiehn, Long & Smith**

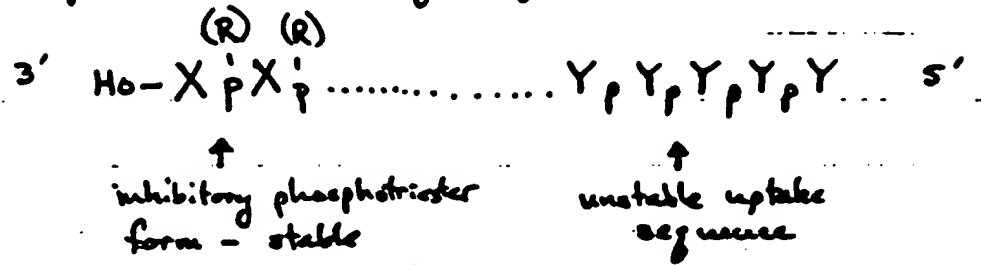
① How well does an oligonucleotide get into a cell if it is synthesized as a phosphotriester or left charged?

② How soluble are phosphotriesters and would that be a problem for either injecting them; clearance by white blood cells, kidney, etc; and would they get to the right place

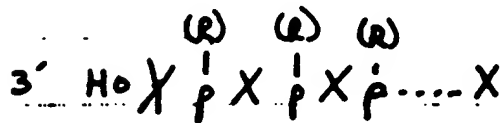
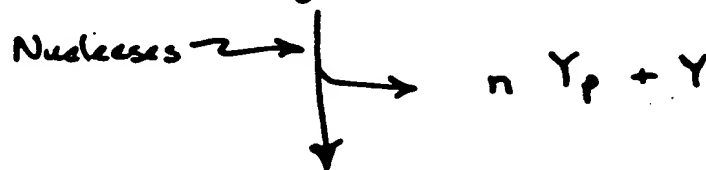
③ It is known that oligonucleotides w/  $2' \rightarrow 5'$  phosphodiester linkages are stable to nucleases. Might use that in place of phosphotriester to stabilize the inhibitor. Problem is that  $2' \rightarrow 5'$  oligo's may not form stable duplex structures - that's not known yet.

④ There may be sequence selectivity to cellular uptake of DNA molecules. This is true for Haemophilus influenzae. If so you could add that sequence

as a relatively unstable oligonucleotide on the 5' side of the inhibitory oligonucleotide.



When this gets taken up into a cell the uptake signal sequence can be cleaved leaving the more stable inhibitory sequence

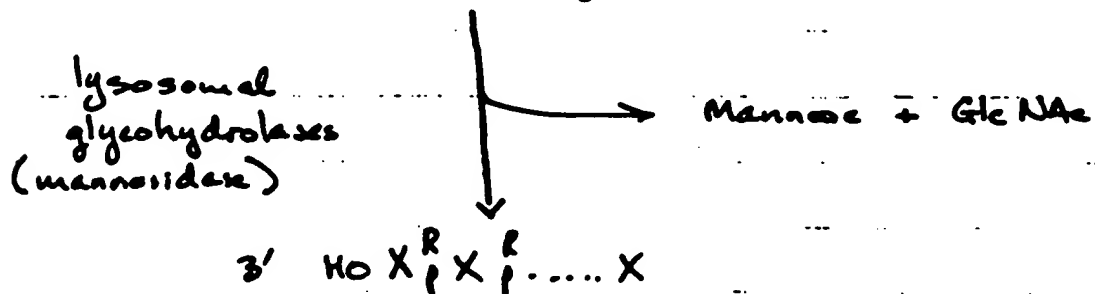


~~Basically~~ This idea could be extended to other types of labile carriers. I don't at this point know enough about uptake of molecules to know the best ones but one such carrier might look like



Once again cells (mammalian cells this time - fibroblasts, liver cells) have specific uptake systems for non-sialylated polysaccharides with a high mannose content

Therefore this material should be taken up just like asialoglycoproteins by pinocytosis into (ultimately) lysosomes where the sugar is degraded leaving the oligonucleotide which can escape to the rest of the cell and subsequently block translation of its target mRNA.



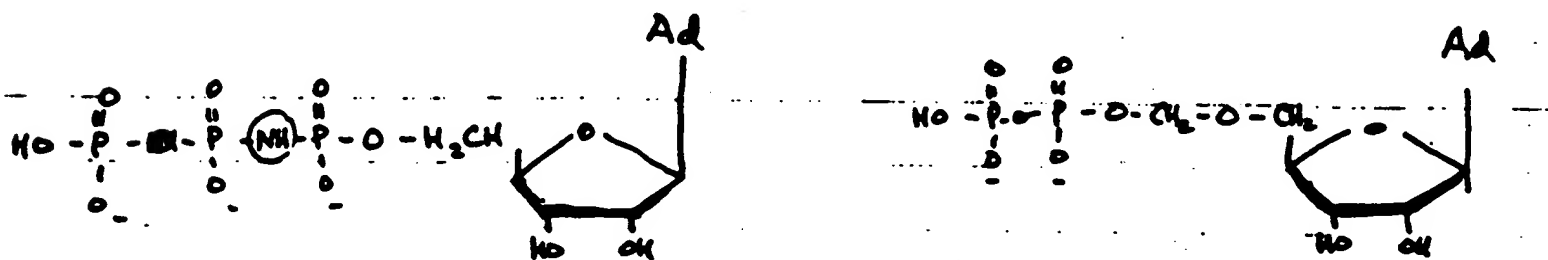
This system would tend to localize the oligonucleotide in liver, since this is the major clearing site for asialoglycoproteins.

I have been trying to think of an acronym for Specific Translation Inhibiting Oligonucleotide Phosphotriester. The only one which seems reasonable is "STOP" molecule. I think I'll be using that abbreviation from now - tell me what you think?

The use of a 2'-5' linkage in place of 3'-5' phosphodiester link seems very interesting. This is the type of linkage produced in cells exposed to interferon. They make oligomers like  $\text{HO} - \text{A} 2' - \text{p} - 5' \text{A} 2' - \text{p} - 5' \text{A} \text{ ppp}$  (an digoribonucleotide)

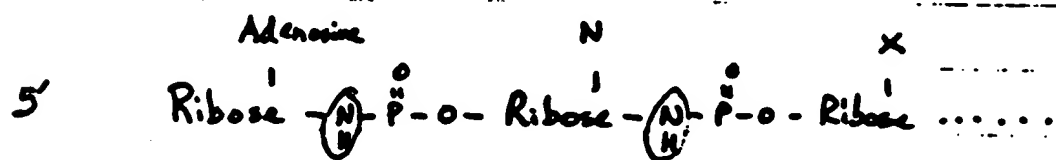
Some new technology would be required to chemically synthesize 2'→5' oligoribonucleotides. I suspect that would take 3-5 years to really work out the details. You might be able to get the basic process in a year or two. If an enzyme exists which does this the process could be much faster.

I just had another thought regarding stable internucleotide linkages. Perhaps one could substitute another linking atom for phosphorous or oxygen analogous to the use of amides in ATP to prevent breakdown (also methylene ether linkages)



"Adenosine triphosphoramidate"      " $\alpha$ -methylene ADP"

The analogous structures would be an oligomer



"Oligonucleotide phosphoramidate"

or

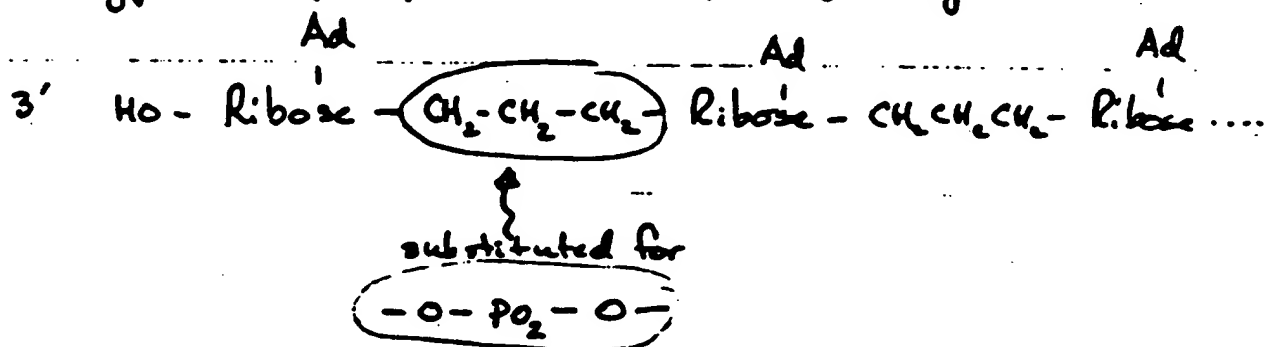
(Could this form helical duplex structures??)  
probably could

3' to 5' or

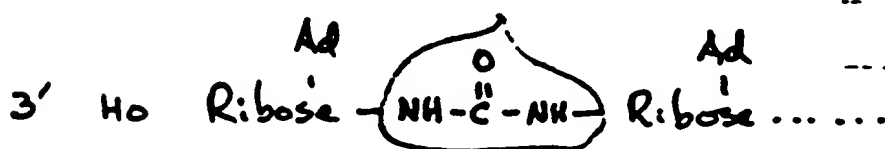
2' to 5' methylene ether linkage

This <sup>exact</sup> structure is not known to occur in nature as far as I am aware. I'm not sure how chemically stable it would be but it looks OK to me. Clearly it is uncharged and therefore less soluble than the phosphoramidate or the 2'→5' phosphodiester.

• You might also be able to make a methylene bridged structure of this type where  $\text{CH}_2$  is substituted for oxygen and phosphorous in normal linkage



The last one for today is an amide bridge substituted for the phosphodiester



this would be more polar and thus more soluble than the methylene or methyl diether form



Since polypeptides can form  $\alpha$  helical structures it is likely that this  $\gamma$  would also form duplex structures. It might be more labile in a cell than some of the other forms since the amide bond could <sup>possibly</sup> be attacked by nonspecific proteases.

Aside from the type of different internucleotide linkages which are possible in the "STOP molecule", one question which has arisen is the antigenicity of polynucleotides. By themselves oligonucleotides are very poorly antigenic. The only known <sup>autoimmune</sup> disease associated with anti-DNA antibodies is systemic lupus erythematosus. However it is possible to raise antibodies to mononucleotides coupled to serum albumin. Thus this problem is possible but unlikely to present a serious objection to the use of STOP.

One question we raised that needs to be answered quantitatively is what is the rate of reaction of STOP with mRNA (i.e. how fast could it begin to block specific mRNA translation) A related question is how much material would have to be injected to inactivate say 1 Kg of virus mRNA in 24 hours?

In order to calculate a rate of reaction of an oligonucleotide of say 20 NT length with an mRNA one can use the formula

$$K_{\text{stop}} = \frac{2 \times K_{\text{cdi}} \times \text{Genome Complexity (cdi)}}{\text{STOP complexity}} \left[ \frac{\text{length of cdi DNA standard}}{\text{length of STOP}} \right]^{-\frac{1}{2}}$$

$$K_{\text{cdi}} = 0.25 \text{ M}^{-1} \text{ sec}^{-1} \quad (\text{second order rate constant})$$

Length cdi = 450 NT for this rate in 0.12 M salt

$$G_{\text{cdi}} = 4.2 \times 10^6 \text{ NT} \quad (\text{NT = base pairs of NT's in cdi genome})$$

$K_{\text{stop}}$  = recessed rate constant expected

$$\text{STOP} = 20 \text{ NT complexity} = 20 \text{ NT length}$$

$$K_{\text{stop}} = \frac{2 \times 0.25 \times 4.2 \times 10^6}{20} \left[ \frac{20}{450} \right]^{-\frac{1}{2}} = 2.21 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$$

In an RNA or ssDNA driven reaction  $K$  is pseudofirst order and has the same value as above

Under these circumstances  $K = \frac{\ln 2}{C_0 t_{1/2}}$

where  $C_0$  = initial & final concentration of STOP and  $t_{1/2}$  is the reaction half time. Thus  $C_0 t_{1/2} = 3.13 \times 10^{-5}$  (M sec). for STOP reacting with an mRNA. If we were to achieve a reasonable intracellular concentration of STOP of say  $5 \mu\text{g/ml}$  of cell volume how fast would this reaction take place?

Or put another way how long would it be before 75% of the mRNA target was complexed with STOP as a hybrid.

(75% rxn is 2 half lives or  $2 \times t_{1/2}$ )

ave NT molecular weight = 315

$5 \mu\text{g/ml} = 15.9 \mu\text{moles/l} = 16 \mu\text{M}$  in NT

(by convention you calculate the concentration of an oligomer or DNA in moles NT/liter)

$C_0 = 16 \mu\text{M}$

$t_{1/2} = 3.13 \times 10^{-5} \text{ M sec}$

$\therefore t_{1/2} = 1.96 \text{ seconds}$  or 4 seconds to 75% reaction

This is clearly fast enough to do the job.

The related question of how much viral mRNA can be inactivated by that amount of STOP molecule. To get  $5 \mu\text{g/ml}$  in all cells (assuming uniform distribution) you would need to inject about 500 mg/100 kg body weight (ie  $\sim \frac{1}{2}$  gm STOP).

500 mg STOP = 79  $\mu\text{moles}$  STOP (20 NT long)

Theoretically 1 molecule of STOP can inactivate 1 molecule of mRNA. Most mRNAs are around 1000 NT long. Thus 500 mg STOP-20 could inactivate 25 gms of viral mRNA.

Since in general there is about 10 x more viral mRNA than DNA, ~~then~~ we would expect  $\frac{1}{10}^{th}$  that amount of viral DNA to be blocked. However a viral genome is about  $3 \times 10^5$  NT long or about 300 times the size of the viral mRNA. Therefore 500 mg STOP could block the replication of 750 gms of viral DNA

$$25 \text{ gm} \times \left(\frac{1}{10}^{th}\right) \times 300 = 750 \text{ gms virus}$$

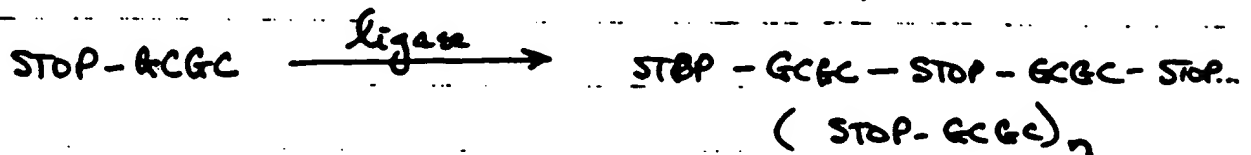
This is far in excess of the amount of viral DNA one might expect to find in an infected human.

I would estimate from this that the minimum effective dose would be much less - at a guess about 1 gm of virus. At this level the required dose of "STOP-20NT" would be 0.7 mg. For a ten fold excess lets say 10 mg. The 75% inactivation time would then be 3.3 minutes (theoretically).

How much could this cost? If we use current retail cost figures as of last August 2 mg of 15 NT oligomer cost \$8500 while 1 mg cost retail \$7500. I am sure this is exorbitant but from the difference between 1 and 2 mg of \$100.00 I would guess that the actual cost in materials is close to \$50 per mg. Since this technique ~~is~~ now automated

that is probably in excess of the real cost. However, using that figure a dose of 10mg would cost \$500.<sup>00</sup> Bulk production could probably cut that to \$50 which would be reasonable.

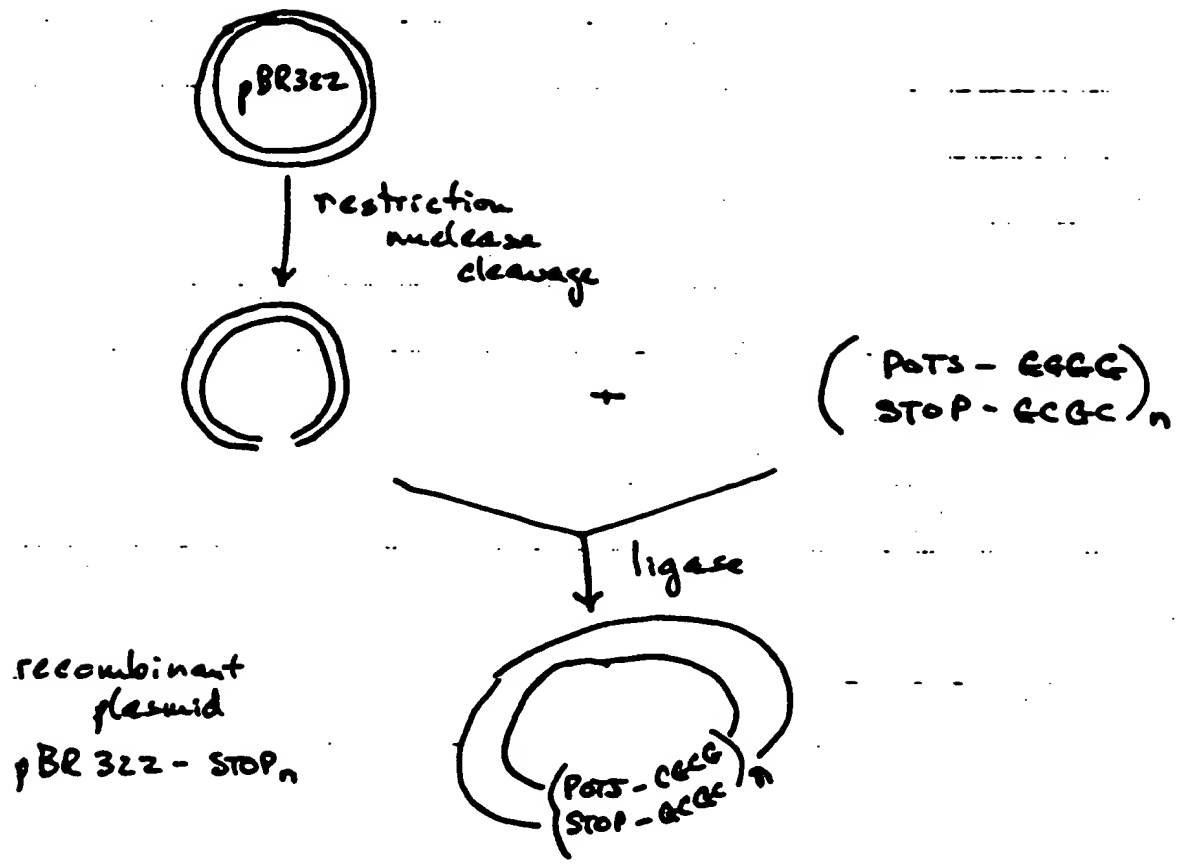
I have thought about ways to produce this material using recombinant technology. The method would be to synthesize a STOP-polymer by blunt end ligation using polynucleotide ligase. The trick would be to leave a restriction nuclease cleavage site at the end of each STOP molecule (eg GCGC) [GCGC is the cleavage sequence for nuclease Hha I] (and Hae II)



This molecule in its double stranded form would be



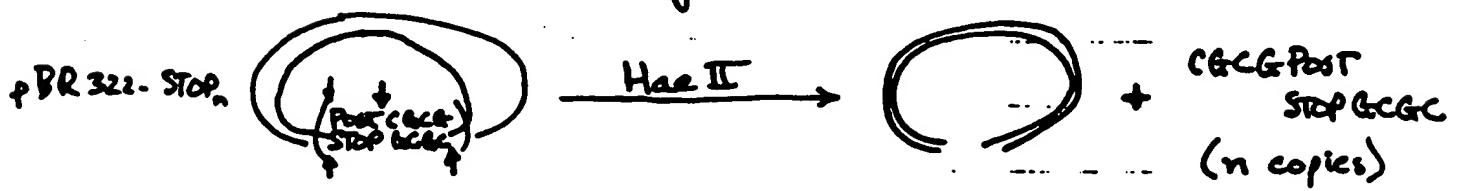
This can be condensed with a high producing plasmid such as pBR 322 to yield a recombinant shown on next page



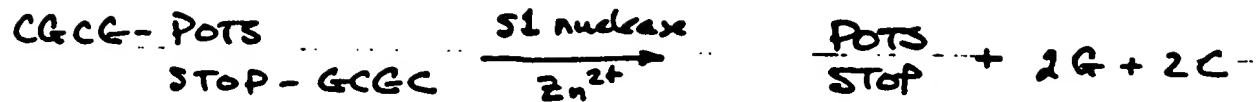
The plasmid pBR 322 - STOP<sub>n</sub> could then be cloned as usual and large amounts grown in E. coli. To isolate STOP the procedure would be as follows:

Std. technique

- 1 Isolate and purify pBR 322 - STOP<sub>n</sub>
- 2 Cleave with restriction nuclease Hae II or Hha I. The best choice will usually be Hae II which will do this:



- 3 Isolate STOP— on a POTS - Sepharose affinity column after removal of GCGG tails by S1 nuclease treatment (specific for single strand DNA)

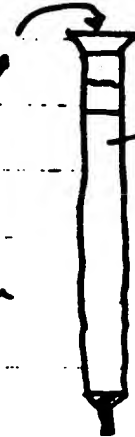


POTS  
STOP

heat  
(to denature  
duplexes)

POTS + STOP

Elute POTS free in  
high salt then elute  
bound STOP with  $\text{H}_2\text{O}$   
(denatures duplex)



POTS affinity  
resin

Hybridizes to  
STOP only

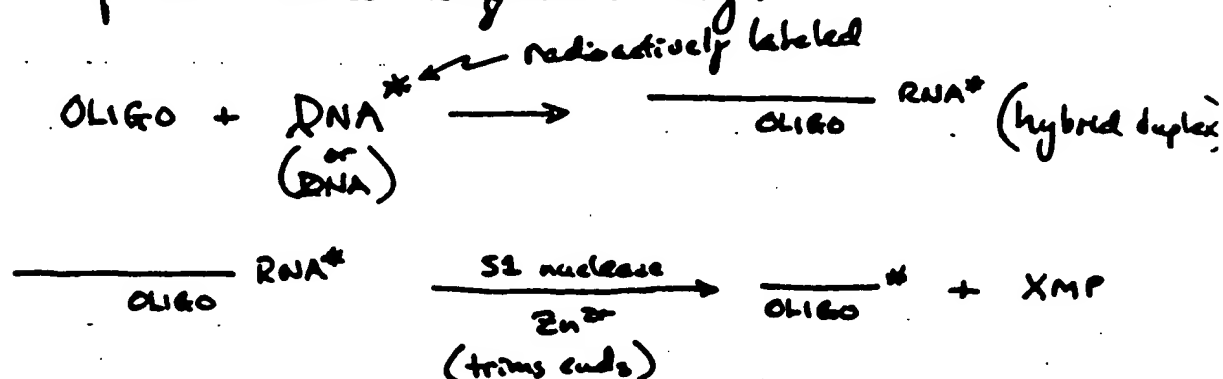
pure STOP

POTS

The cost of STOP produced essentially in this manner (Note: there must be a triester formation step just before denaturing POTS STOP above) would be analogous to the cost of producing insulin via recombinant bugs. That cost is about 1¢/mg. Since in a cell there is generally speaking 10-100x more protein than DNA a rough estimate of the cost would be at most about \$1<sup>00</sup>/mg STOP. Thus a 10mg treatment dose would cost ~ \$10<sup>00</sup>/10mgs STOP.

(Not bad for off the top so far, eh?)

The idea I had for using oligonucleotides to detect specific microorganisms (eg Gonococcus) is analogous to radioimmunoassay. The basic principle is that a hybrid can be specifically formed between an oligonucleotide and a particular DNA or RNA species, which is absolutely specific to that particular sequence. This can be competed out by unlabeled DNA or RNA of the same sequence only.

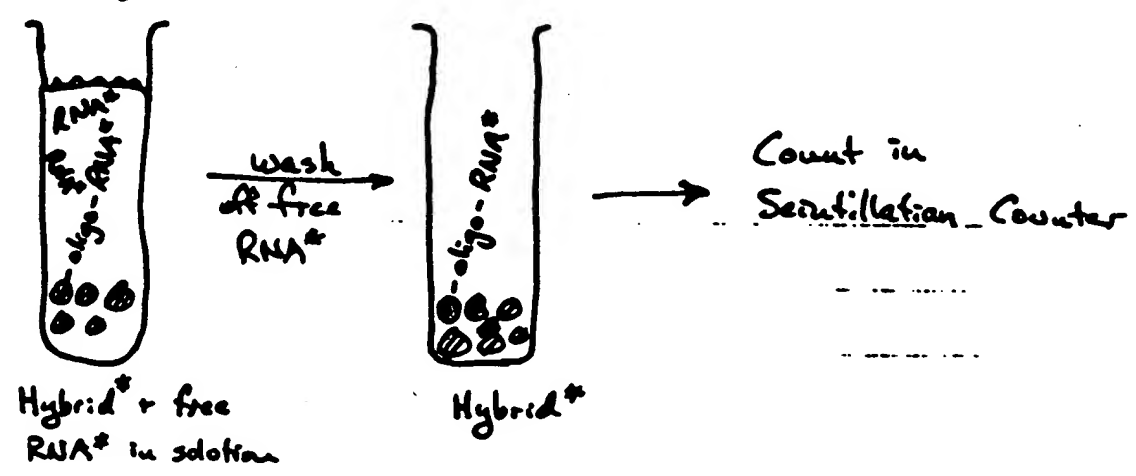


The hybrid can be measured by binding to DEAE paper or hydroxylapatite chromatography.

In the presence of cold, unlabeled specific RNA (or DNA) the oligonucleotide would be competed for in forming hybrids. Thus less radioactive hybrid would be formed. The degree of reduction would be a measure of the amount of competing RNA (or DNA) present, thus allowing estimation of the amount of organism present in say a vaginal smear or sputum sample.



The calculations for the rate of reaction, sequence excess required, sensitivity, cost and complication are rather involved so I would like to put that off to a future letter. However, one last note on this and then I'll quit. The reaction could be run using oligomer bound to a solid support (eg Glass bead  $\rightarrow$  oligo) in a rapid reaction system known as PERT (phenol emulsion reassociation technique) which is 10,000 times faster than normal solution hybridizations and does not require purification of DNA prior to use. The idea would then be to put the sample directly into the phenol emulsion in a small tube containing <sup>glass bead</sup>  $\rightarrow$  oligo + RNA\* and shake for an hour or so. The beads could then be rinsed several times with saline and counted directly in a scintillation counter



I know that's a lot of stuff, but you did say to put down my thoughts so there they are. I'll provide more detail later. I hope this will do for a start.

I am also including a bibliographic search done on "synthetic oligonucleotides" in Bio Abstracts and copies of some articles for a recent Science which may interest you regarding oligonucleotides.

If you plan on coming to San Diego soon let me know so we can get together and talk. Otherwise I'll be in touch when I can.

Best regards

Rich